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DIFFERENCES IN THE FLOCCULATION MECHANISM OF *Kluyveromyces marxianus* AND *Saccharomyces cerevisiae*.

M. J. Sousa, J. A. Teixeira, M. Mota*
Centro de Engenharia Química da Universidade do Porto
Rua dos Bragas 4099 Porto Codex, Portugal

SUMMARY

A study of the flocculation mechanism of a *Kluyveromyces marxianus* strain, as compared with a strain of *Saccharomyces cerevisiae*, is described. The involvement of cell wall proteins in the yeast's flocculation mechanism was studied by methyl-esterification or pepsin or acid phosphatase incubation of the cells. The influence of several ions on the flocculation mechanism was assayed. The obtained results indicated that the structure and/or the spatial arrangement of the cell wall groups involved in flocculation are not the same in *K. marxianus* as in *S. cerevisiae*.

INTRODUCTION

In recent years, flocculation has been receiving a growing attention both from industry and from research. This is due to the importance of flocculation as a high productivity/low cost separation process (Prince and Barford, 1982; Netto *et al.*, 1985; Teixeira *et al.*, 1990) and as a relevant phenomenon for classical fermentation industries such as brewing (Greenshields and Smith, 1971; Stewart and Russel, 1986).

The yeast flocculation is described as a cell wall interaction (Eddy and Rudin, 1958; Masschelein *et al.*, 1963). Mill (1964) suggested that Ca^{2+} mediated ionic bridges between proteins of adjacent cells. According to this author hydrogen bonds between carbohydrates could be possible, stabilizing the Ca^{2+} bridges. Other authors (Taylor and Orton, 1975; Miki *et al.*, 1982) proposed the existence of a bilateral connection between protein and mannans in the surface of each cell, mediated by Ca^{2+} ions binded to the protein side.

Although there is a general agreement on the involvement of cell wall mannans and proteins on flocculation, it is not clear what kind of functional groups are directly implicated. Most of the authors point that carboxyl groups play an important role in flocculation (Stewart *et al.*, 1976), while others refer to the importance of phosphate groups (Lyons and Hough, 1970; Lyons and Hough, 1971).

However, the main studies on yeast flocculation only report results obtained with *Saccharomyces* strains. Few works (Hussain *et al.*, 1986; Mahmood et al., 1987; Teixeira *et al.*, 1989) deal with flocculation yeasts belonging to other genera.

Also, there is no general agreement in the ions that are required for cell-cell interaction. Most of the authors agree on the importance of calcium (Mill, 1964; Lyons and Hough, 1970; Taylor and Orton, 1975; Amri *et al.*, 1979; Stratford, 1989), due to its

specificity, to promote flocculation. This opinion is not shared by other authors (Stewart and Goring, 1976; Nishihara *et al.*, 1982) who claim that Mg^{2+}, Mn^{2+} as well as other ions are as effective as calcium.

In this work, the effect of several ions on the flocculation ability of two yeast strains belonging to different genera, *K. marxianus* and *S. cerevisiae*, is studied, using both whole cells and cell walls. The last technique was employed in order to avoid possible ion fluxes from inside the cells. Also, several chemical and enzymatic treatments were performed on the cell, in order to elucidate whether the kind of functional groups directly involved in flocculation were the same for both strains.

MATERIALS AND METHODS

Strains: The flocculent *Kluyveromyces* strain used was isolated from a non flocculating one - *K. marxianus* ATCC 10022. The *Saccharomyces* flocculent strain used was *S. cerevisiae* NRRL Y265. The *K. marxianus* flocculent cells were taken from the continuous bioreactor where flocculation was induced (Mota and Teixeira, 1990). The medium fed to the reactor was a lactose (60 g/l) based medium, complemented with yeast extract (1 g/l), potassium di-hydrogen phosphate (5 g/l), ammonium sulphate (2 g/l) and magnesium sulphate (0.4 g/l). For the *S. cerevisiae* strain, cells were grown for 48 hours in 1 liter Erlenmeyer flasks, containing the previously described semi-synthetic medium using glucose (100 g/l) as carbon source instead of lactose.

Preparation of cell walls: Cells were harvested by centrifugation, and washed twice with 15 g/l NaCl pH 2, and twice with EDTA 200 mM. The pellet was mixed with 200 mM EDTA solution and glass beads in the ration 1:2:1 (wet wt/v/v), and cells were broken in a Braun homogenizer (MSK). The cell walls were collected, by centrifugation at 1500 g, 10 min., and then washed as follows: 5 times with water; twice with 15 g/l NaCl; twice with 7.5 g/l NaCl; and 5 times with water.

Measurement of flocculation ability: The flocculation ability of the strains was assayed using a modification of the Helm sedimentation test (Stewart, 1975). Cells were previously deflocculated: yeast cells were separated from the liquid phase by centrifugation and then washed 3 times with each of the following solutions: 15 g/l NaCl solution at pH 2; 200 mM EDTA solution; and ultrapure water. Cell walls were washed 3 times with 200 mM EDTA solution, and 3 times with ultrapure water. After this treatment, cells or cell walls were suspended in a 1 mM aqueous solution of the desired cation (under chloride form) adjusted to pH 4 with HCl. The suspension was then placed in a 25 ml cylinder and inverted four times for homogenization. At defined intervals, samples were taken from a fixed position (level corresponding to 20 ml), and biomass concentration measured spectrophotometrically at 620 nm. At the end of the assays the pH variation was no higher than 0.2.

The obtained biomass concentrations were normalized against the initial biomass concentration (taken as 100%) and the sedimentation profile was plotted as the percentage biomass in suspension with time. The initial cell dry weight concentration in the cylinder was 4 g/l. The results presented are the average of at least two independent experiments.

All the solutions were made with ultrapure water. All the glassware was washed in an ultrasound bath, treated with a sulpho-chromic mixture, washed with HCl 1 M and rinsed three times with ultrapure water.

Chemical and enzymatic modification of yeast cell walls: The chemical treatment was done by incubating 100 mg (dry weight) of flocculating cells in 100 ml of a 0.1 N HCl methanolic solution for 24 hours at 30°C. For pepsin treatment cells (100 mg dry weight) were incubated with 100 mg of pepsin in 50 ml of an aqueous HCl solution at pH 2 for one hour at 37°C. For acid phosphatase treatment cells (60 mg dry weight) were incubated with 0.8 mg of acid phosphatase in 12 ml of a 15 g/l NaCl aqueous solution at 30°C, for one hour. A high NaCl concentration was used in order to ensure cell deflocculation. The acid phosphatase activity was assayed by measuring DNPP's hydrolysis

by acid phosphatase, using the same experimental conditions (pH, temperature and NaCl concentration) as for the cells. Chemically or enzymatically treated cells and non-treated cells (controls) were tested for their flocculation ability, as described above, in the presence of $CaCl_2$ 1 mM.

Influence of several cations in the flocculation: The following ions (under chloride form) were used: Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Sr^{2+}, Sn^{2+}, Al^{3+} and Ce^{3+}. The influence on the flocculation ability of the yeast was measured according to the previously described modification of the Helm sedimentation test.

RESULTS AND DISCUSSION

Modification of yeast cell walls

The effect of chemical and enzymatic treatments in the flocculation ability of two yeast strains - *K. marxianus* and *S. cerevisiae* - was studied. Treated cells were compared with non-treated cells of the same strain. The differences in flocculation between the two strain controls is not taken into consideration here.

The incubation of both yeast strains in methanol/HCl solution, strongly reduced their flocculation ability. In a sedimentation test, after a 5 minute settling period, more than 98 % of the treated yeast cells remained in suspension (Figs. 1 and 2).

Also, the incubation of flocculent cells with pepsin eliminated the flocculation ability both for *S. cerevisiae* and *K. marxianus* (Figs. 1 and 2).

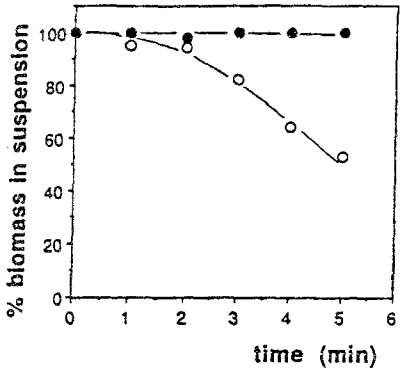

Fig. 1 - Sedimentation profiles of *K. marxianus* cells: (O) - non-treated cells; (●) - acid phosphatase, methanol/HCl or pepsin treated cells.

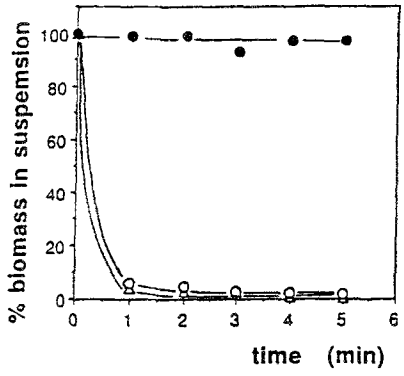

Fig. 2 - Sedimentation profiles of *S. cerevisiae* cells: (Δ) - non-treated cells; (O) - acid phosphatase treated cells; (●) - methanol/HCl or pepsin treated cells.

For the *Saccharomyces* strain, no reduction was observed in the flocculation ability after incubation with acid phosphatase. The sedimentation profiles of the treated and non treated cells were identical (Fig. 2). The results obtained for *K.marxianus* indicated a small reduction on the flocculation capacity of the enzyme treated cells. As can be seen in Fig. 1, after a 5 minute settling period no sedimentation was measurable, although some small flocs were visible in the cylinder.

The results obtained after pepsin digestion of yeast cell walls clearly demonstrated that there is a protein involved in the flocculation mechanism for both *Kluyveromyces marxianus*

and *Saccharomyces cerevisiae* strains. Our results agree with those reported by other authors who worked with *Saccharomyces* strains, and reinforce the hypothesis that a protein is involved in the flocculation mechanism of the *K. marxianus* strain (Teixeira *et al.*, 1989).

The different behavior of both flocculent strains after incubation with acid phosphatase suggests that the involvement of phosphate groups in flocculation is different. In what concerns the *Saccharomyces* strain there is no evidence of the involvement of cell wall phosphate groups in the flocculation mechanism, since no difference in the sedimentation rate for treated and non treated cells was observed. The *Kluyveromyces* strain, however, showed a reduction in the sedimentation capacity, suggesting that phosphate groups play a role in flocculation. This is also confirmed by the larger content of phosphate groups on the cell wall of the flocculent *K. marxianus* strain, when compared with the non flocculent one (Teixeira *et al.*, 1989).

Methanol/HCl treatment esterifies carboxyl and phosphate groups. These esterification can be reversed by hydrolysis with 8 M urea, without destroying the flocculation ability of the cells (Nishihara and Ueda, 1979). Since, unlike methanol/HCl treatment, phosphatase treatment of *S. cerevisiae* cells had no effect on their flocculation ability, the carboxyl groups must be the ones involved in the flocculation mechanism of these yeast which is in agreement with most of the authors (Taylor and Orton, 1975; Stewart *et al.*, 1976; Miki *et al.*, 1982; Nishihara *et al.*, 1982). For *K. marxianus* cells the methanol/HCl treatment does not allow to conclude about the carboxyl groups involvement, since phosphate treatment also inhibited flocculation.

Influence of several cations in the flocculation ability of both strains.

The influence of several cations on the flocculation of *K. marxianus* and *S. cerevisiae* was studied by following the settling profiles of yeast cells in the presence of each cation. Seven divalent and two trivalent cations were used. For *K. marxianus* cells (Fig. 3) Ca^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+} or Mg^{2+} had all similar effects, being able to promote flocculation. Nevertheless, they were not as efficient as Fe^{2+} or Sn^{2+}, which were the most effective ions tested. The trivalent ions, Ce^{3+} and Al^{3+}, were both capable of promoting flocculation, showing Ce^{3+} a slight lower effect than Al^{3+}.

S. cerevisiae cells had a different behavior in the presence of these ions. Indeed, Ce^{3+} was unable to promote flocculation, even after a 10 min. settling period, whilst cells sediment quite well in the presence of Al^{3+} (Fig. 4). When comparing the effect of divalent ions on *S. cerevisiae* cells, two distinct sedimentation profiles can be distinguish. One, for Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} or Fe^{2+}, where very fast and extensive sedimentation occurs, and another where the sedimentation is slower, which occurs in the presence of Sn^{2+} or Sr^{2+} . Of this two, Sn^{2+} was the one which promoted a more complete flocculation.

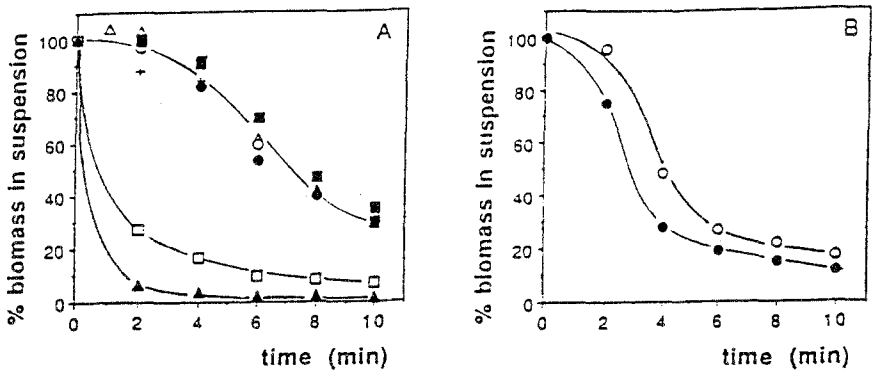

Fig. 3 - Sedimentation profile of *K. marxianus* in the presence of: A - several divalent ions: (⊡) Mg^{2+}, (△) Ca^{2+}, (●) Mn^{2+}, (□) Fe^{2+}, (○) Co^{2+}, (+) Sr^{2+}, (▲) Sn^{2+}; B - two trivalent ions (●) Al^{3+}, (○) Ce^{3+}

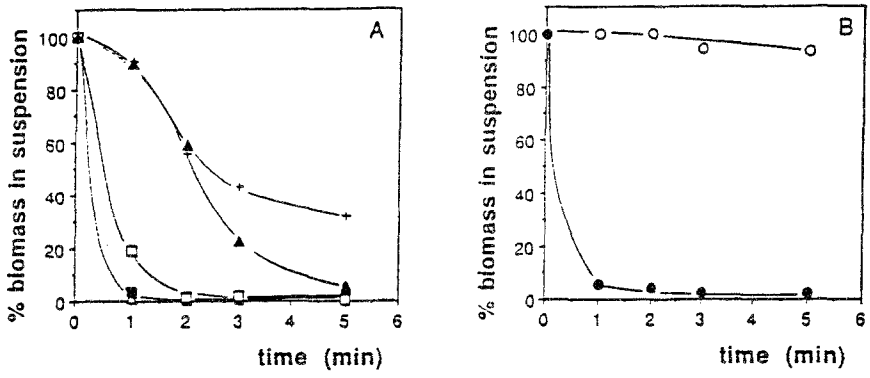

Fig. 4 - Sedimentation profile of *S. cerevisiae* in the presence of: A - several divalent ions: (⊡) Mg^{2+}, (△) Ca^{2+}, (●) Mn^{2+}, (□) Fe^{2+}, (○) Co^{2+}, (+) Sr^{2+}, (▲) Sn^{2+}; B - two trivalent ions: (●) Al^{3+}, (○) Ce^{3+}

Like Nishihara *et al.* (1982), we found some inhibition of flocculation in the presence of Sr^{2+} for *S. cerevisiae*. In contrast no inhibition effect on flocculation was observed with Sr^{2+} for *K. marxianus*. Another significant difference in the flocculation behavior between the two strains was their sedimentation rates. Indeed, for *S. cerevisiae* sedimentation was much faster than for *K. marxianus*. The only exception was observed for Sn^{2+}. This ion promoted flocculation even in non-flocculent cells (Nishihara *et al.*, 1982).

In order to elucidate if the effect of the cations was due to the direct action on the cell wall or due to the induction of a Ca^{2+} efflux from the cells, the same assays were also performed using isolated cell walls. All the results were similar to those obtained with whole cells (data not shown). This clearly indicates that the ions affected directly the cell wall, and hence there is not a specific requirement for Ca^{2+} ions in flocculation. Our observations are in opposition to some authors who propose that Ca^{2+} is specifically needed

for flocculation, and that other ions act by enhancing calcium leakage (Stratford, 1989 and Kuriyama *et al.*, 1990).

The results obtained with the effects of the several tested ions on flocculation of both *K. marxianus* and *S. cerevisiae* indicate that the interaction mechanism is different for each strain. An attempt to correlate yeast cell flocculation with chemical bonding properties of the different equally charged ions, namely, ionic radius, electronegativity, coordination number, did not justify the differences found between strains. The results obtained with chemical and enzymatic treatments, further support the differences in flocculation mechanisms in the two yeasts. Some hypothesis may be presented to explain this behavior: there may be a different spatial arrangement of the functional groups involved in flocculation or cell wall groups involved in flocculation may have different chemical composition.

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